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Short Communication

Encapsulated *Lactococcus lactis* with enhanced gastrointestinal survival for the development of folate enriched functional foods

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HIGHLIGHTS

- Two probiotic *Lactococcus lactis* strains were encapsulated.
- Co-encapsulation and hybrid entrapment were employed for encapsulation.
- Encapsulation increased their survival in simulated gastrointestinal conditions.
- Comparable folate production was shown by free and encapsulated probiotics.
- Encapsulated probiotics were used to develop folate fortified foods.

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ABSTRACT

Two lactic acid bacteria (LAB) isolated from cow's milk were identified as *Lactococcus lactis* strains and designated as *L. lactis* CM22 and *L. lactis* CM28. They were immobilised by co-encapsulation using alginate and mannitol and by hybrid entrapment with skim milk, glycerol, $CaCO_3$ and alginate. The encapsulated cells survived better in simulated gastrointestinal conditions compared to the free cells. The percentage survival of probiotics encapsulated by hybrid entrapment method was 62.74% for *L. lactis* CM22 and 68% for *L. lactis* CM28. Studies to check their efficacy in fermentative fortification of skim milk and ice cream revealed an enhancement in folate level.

1. Introduction

Probiotics, live microbes which when administered play a great role in maintaining host health by improving intestinal balance (Fuller, 1989). The reported health benefits of probiotics include immunomodulation, maintaining gut flora, alleviation of lactose intolerance, anti cancer activity, cholesterol lowering effect (Divya et al., 2012), etc. Lactic acid bacteria (LAB) are the prominent probiotics known to have beneficial effects. The growing awareness among consumers for what they eat lead to the development of probiotic functional foods which are enriched with health promoting ingredients. Some of the nutraceuticals produced by LAB are B group vitamins, low calorie sugars and exopolysaccharides (Divya et al., 2012). It is necessary that the probiotics survive the acidic conditions of the stomach and reach the small intestine in a viable state to colonize the host. Hence, an adequate level of viable bacteria in the food product at an appropriate daily dose ($\geqslant 10^7$ CFU/g) is essential to exert its beneficial effect on host (Charalampopoulos et al., 2002). When probiotics are administered orally they must be protected from various factors like the acidic pH of the stomach, digestive enzymes, bile salts, etc. These factors limit the survival of probiotics thus preventing them from exerting their positive effects.

Microencapsulation provides a physical barrier to the probiotics against the harsh environmental conditions and gastrointestinal passage thereby increasing the number of viable cells reaching the small intestine (Kailasapathy, 2002). It is the entrapment of probiotic bacteria into food grade matrices like alginate, starch, milk proteins, etc. It also aids in the passage of metabolites and controlled release of the probiotics. Calcium alginate is the most widely used encapsulation matrix for probiotics due to the relatively simple and cheap procedure and non toxic nature. But the alginate beads are susceptible to acidic environment and this result in crackling and loss of mechanical strength of the beads (Mortazavian et al., 2007). Also, alginate is unstable in presence

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of chelating agents like lactate which have affinity towards calcium and this could result in the disintegration of beads during lactic acid fermentation thereby releasing the probiotics before reaching the lower intestinal tract or colon (Smidsrod and Skjak-Braek, 1990). However, blending of alginate with certain encapsulating additives such as prebiotics, milk proteins and starch can increase its mechanical and chemical stability.

The aim of the present study was to increase the gastrointestinal survival of the two folate producing *Lactococcus lactis* strains by different encapsulation techniques and to formulate folate enriched functional foods by using the encapsulated probiotics.

2. Methods

Two LAB strains isolated from cow's milk with probiotic characteristics (Gangadharan et al., 2010) identified as *L. lactis* strains and designated as *L. lactis* CM22 (NCBI accession no: KJ742708) and *L. lactis* CM28 (NCBI accession no: KJ676682) were used for the encapsulation studies. The cultures were maintained in M17 medium supplemented with 0.5% glucose and sub cultured every 2 weeks. Folic acid standards were procured from Sigma (MO, USA). All media and reagents were purchased from Himedia, India, unless otherwise mentioned.

2.1. Encapsulation of the LAB strains

The encapsulation was carried out by extrusion method by using two different encapsulation matrices. Co-encapsulation was done by a modified method of Dianawati and Shah (2011). In this method, the encapsulation matrix consisted of alginate (2.5% w/v) and mannitol (2.5% w/v) solution. In hybrid entrapment method (Reyed, 2006), an encapsulation medium containing 10% skim milk, 5% glycerol, 0.1% $CaCO_3$ along with 2.5% sodium alginate was used. The beads were prepared by extrusion method and the prepared beads containing individual culture were then lyophilized and stored in refrigerator.

2.2. Survival of encapsulated probiotics in simulated gastric juice (SGJ)

SGJ was prepared by dissolving NaCl (9 g/L) and pepsin (3 g/L) and the pH was adjusted to 2.5 and 3 using HCl. 1 g probiotic beads were added to 10 mL SGJ (pH 2.5 and 3). Samples were collected at 0, 1 and 2 h, homogenized in phosphate buffer, serially diluted with saline and the appropriate dilutions were plated onto M17 plates supplemented with 0.5% glucose.

2.3. Survival of encapsulated probiotics in simulated intestinal juice (SIJ)

SIJ was prepared by dissolving 1.5% bile salts (bile salts mixture) in 0.05 M KH_2PO_4 and pH was adjusted to 7. 1 g encapsulated beads were transferred to 10 mL SGJ of pH 3 and incubated at 37 °C for 1 h. The beads were then transferred to 10 mL SIJ of pH 7 and incubated at 37 °C for 2 h. Sampling, dilution and plating were done as described before. Results were expressed as log CFU/mL. Survival of free cells in SGJ and SIJ was also determined as control.

2.4. Folate fortification of ice cream and skim milk using encapsulated probiotics

Skim milk medium optimized for folate production by *L. lactis* CM22 (SM2 medium) and *L. lactis* CM28 (SM1 medium) were used for the fortification studies. The composition of SM1 was skim milk (4%), PABA (100 μmol/L), glutamate (75 μmol/L), glycine (6 μmol/L), methionine (6 μmol/L), mannitol (0.6%), sodium ascorbate (0.2%) and that of SM2 medium was skim milk (4%), PABA (75 μmol/L), glutamate (75 μmol/L), glycine (6 μmol/L), methionine (6 μmol/L), mannitol (0.8%), sodium thioglycolate (0.2%). 1 g probiotic beads of respective cultures prepared by hybrid entrapment method were added to corresponding skim milk medium (10 mL) and incubated at 37 °C for 10 and 15 h under static condition. Free cells of *L. lactis* CM22 and *L. lactis* CM28 (1% inoculum, i.e., $\sim 10^9$ CFU/mL) were also inoculated into 10 mL SM2 and SM1 medium respectively and incubated at 37 °C for 8 h. Similarly, 1 g beads of each culture were added to 10 mL ice cream and allowed to ferment at 37 °C for 10 and 15 h without shaking. Free cells (1%) of *L. lactis* CM22 and *L. lactis* CM28 were also inoculated into ice cream and incubated at 37 °C for 8 h at static condition.

2.5. Folate analysis

Folate quantification was carried out by microbiological assay using the folate auxotroph *Lactobacillus casei* NCIM2364 as the indicator organism as described before (Divya and Nampoothiri, 2014; Horne, 1997).

All the experiments were carried out in triplicates and the results are expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Survival of free and encapsulated probiotics in simulated gastrointestinal conditions

In order to find out the survival rate of probiotics on oral administration, the free as well as encapsulated probiotics were tested for stability in SGJ (pH 2.5 and 3) and SIJ (pH 7). The survival of encapsulated *L. lactis* CM22 was higher than that of the free cells. There was less than a log unit reduction (9.85 ± 0.07 to 8.92 ± 0.11 log CFU/mL) in the viability of cells encapsulated by hybrid entrapment method where as in free cells the viability was reduced to more than three log units (8.93 ± 0.24 to 5.3 ± 0.22 log CFU/mL) in SGJ of pH 2.5. At pH 3 also the encapsulated cells survived better than the free cells. Similarly, encapsulation improved the survival of *L. lactis* CM28 at pH 2.5 and 3. When compared to co-encapsulation, hybrid entrapment was found to be slightly superior. The results obtained from the survival in SIJ (pH 7) also revealed that co-encapsulation and hybrid entrapment method effectively increased the survival of *L. lactis* CM22 and *L. lactis* CM28 (Fig. 1A and B).

Based on these results the percentage survival of the free and encapsulated probiotics was calculated (Fig. 1C). After incubation in SGJ and SIJ, 51.1% free *L. lactis* CM22 cells were viable whereas 56.34% and 62.74% cells remained viable in case of co-encapsulation and hybrid entrapment. For *L. lactis* CM28, hybrid entrapment retained 68% viability while co-encapsulation resulted in 61.1% survival and for free cells the survival was 55.8%.

The survival of encapsulated probiotics is dependent on a number of factors such as concentration of the polymer used, capsule size, composition ,etc. Dianawati et al. (2012) reported that sugar alcohols like sorbitol and mannitol interact with the polar site of phospholipid bilayer thereby providing protection to the probiotics. When milk proteins were used as encapsulating medium along with alginate the highly dense gel formed provided a favorable milieu for the probiotics. The pH inside the gel matrix will be higher than outside thus providing protection to the probiotics (Heidebach et al., 2009). In a previous study by Guérin et al. (2003) when a combination of alginate, pectin and whey protein was used to encapsulate *Bifidobacterium* cells there was considerable improvement in the survival in SGJ and SIJ.

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